INSTRUCTIONS

Human VEGFA ELISA Kit

EH2VEGF EH2VEGF2 EH2VEGF5

2408.2

Number	Description
EH2VEGF	**Human VEGFA ELISA Kit,** sufficient reagents for 96 determinations
EH2VEGF2	**Human VEGFA ELISA Kit,** sufficient reagents for 2 × 96 determinations
EH2VEGF5	**Human VEGFA ELISA Kit,** sufficient reagents for 5 × 96 determinations

Kit Contents	EH2VEGF	EH2VEGF2	EH2VEGF5
Anti-human VEGFA Precoated 96-well Strip Plate	1 each	2 each	5 each
Lyophilized Recombinant Human VEGF Standard	2 vials	4 vials	10 vials
Standard Diluent	14mL	2 × 14mL	5 × 14mL
30X Wash Buffer	50mL	2 × 50mL	5 × 50mL
Biotinylated Antibody Reagent	12mL	2 × 12mL	5 × 12mL
Streptavidin-HRP Concentrate	75µL	2 × 75µL	5 × 75µL
HRP Dilution Buffer	14mL	2 × 14mL	5 × 14mL
TMB Substrate Solution	13mL	2 × 13mL	5 × 13mL
Stop Solution, contains 0.16M sulfuric acid	13mL	2 × 13mL	5 × 13mL
Adhesive Plate Covers	6 each	12 each	30 each

For research use only. Not for use in diagnostic procedures.

Storage: For maximum stability, store in a non-defrosting -20ºC freezer and refer to the expiration date for frozen storage on the label. Alternatively, store at 2-8ºC and refer to the expiration date for refrigerated storage. Once thawed, store at 4ºC until the expiration date for refrigerated storage. Kit is shipped on dry ice.

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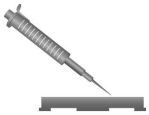
Introduction

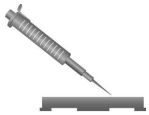
The Thermo Scientific™ Human VEGFA ELISA Kit measures human VEGFA in serum, heparin and sodium citrate plasma, and culture supernatants. The microplate provided is coated with anti-human $VEGF_{165}$ antibody to capture VEGF in standards and samples. After nonbound proteins are removed, a biotinylated detection antibody is added and binds to a second epitope-binding site on the VEGF. Excess detection antibody is removed and streptavidin-horseradish peroxidase is added with TMB to produce colorimetric signal.

Procedure Summary



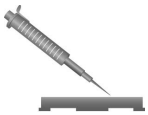
1. Add 50µL of Standard diluent to each well. Immediately add 50µL of Standards or samples to each well.

2. Cover plate. Incubate at room temperature (20-25ºC) for 2 hours.

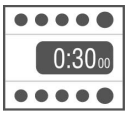


3. Wash plate THREE times. Add 100µL of biotinylated detection antibody.

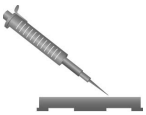
4. Cover plate. Incubate at room temperature (20-25ºC) for 1 hour.



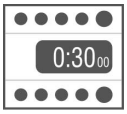
5. Wash plate THREE times. Add 100µL of prepared Streptavidin–HRP to each well.



6. Cover plate. Incubate at room temperature (20-25ºC) for 30 minutes.



7. Wash plate THREE times. Add 100µL of TMB Substrate Solution.



8. Develop plate in the dark at room temperature for 30 minutes.

9. Stop the reaction by adding 100µL of Stop Solution to each well.

10. Measure absorbance on a plate reader at 450nm minus 550nm.

11. Calculate results using graph paper or curve-fitting statistical software.

Additional Materials Required

- Precision pipettors with disposable plastic tips to deliver 5-1000µL and plastic pipettes to deliver 5-15mL
- Glass or plastic 2L container to prepare Wash Buffer
- Squirt wash bottle or an automated 96-well plate washer
- 1.5mL polypropylene or polyethylene tubes to prepare standards – do not use polystyrene, polycarbonate or glass tubes
- Disposable reagent reservoirs (Product No. 15075)
- Microcentrifuge
- 15mL plastic tube to prepare Streptavidin-HRP Solution
- A standard ELISA reader for measuring absorbance at 450nm and 550nm. If a 550nm filter is not available, the absorbance can be measured at 450nm only. Refer to the instruction manual supplied with the instrument being used.
- Graph paper or a computerized curve-fitting statistical software package

Precautions

- **All samples and reagents must be at room temperature (20-25°C) before use in the ELISA.**
- Review all instructions carefully and verify all components against the kit contents list (page 1) before beginning.
- Thaw samples at room temperature. Do not use a water bath to thaw samples.
- When preparing standard curve and sample dilutions in culture medium, use the same medium used to culture the cells. For example, if RPMI with 10% fetal calf serum (FCS) was used to culture cells, then use RPMI with 10% FCS to dilute the standard and samples. DO NOT USE RPMI WITHOUT SERUM SUPPLEMENT.
- To avoid cross-contamination, always use a new disposable reagent reservoir and new disposable pipette tips for each transfer. Also, use a new adhesive plate cover for each incubation step.
- Once reagents have been added to the plate, take care NOT to let the plate dry at any time during the assay.
- Avoid microbial contamination of reagents.
- Avoid exposing reagents to excessive heat or light during storage and incubation.
- Discard unused ELISA kit components after assay completion. Do not mix reagents from different kit lots.
- Do not use glass pipettes to measure the TMB Substrate Solution. Do not contaminate the TMB Substrate Solution; if it is blue before use, DO NOT USE THE SOLUTION.
- Individual components can contain antibiotics or preservatives. Wear gloves while performing the assay to avoid contact with samples and reagents. Follow proper disposal procedures.

Additional Precaution for the 5-plate Kit

- Dispense, pool, and equilibrate to room temperature only the reagent volumes required for the number of plates being used. Do not combine leftover reagents with those reserved for additional plates.

Sample Preparation

A. Sample Handling

- Serum; EDTA, heparin plasma; and culture supernatants may be tested in this ELISA.
- 50µL per well of serum, plasma or culture supernatant is required.
- Store samples at 2-8°C and assay within 24 hours. For long-term storage, aliquot and freeze samples at -70°C.
- Avoid repeated freeze-thaw cycles when storing samples.
- Test samples and standards must be assayed in duplicate or triplicate each time the ELISA is performed.
- Mix samples by gently inverting tubes.
- If samples are clotted, grossly hemolyzed, lipemic or microbially contaminated, or if there is any question about the integrity of a sample, make a note on the template and interpret the results with caution.

B. Sample Dilution

- If the human VEGF concentration from the sample will exceed the highest point of the standard curve (i.e., 2000pg/mL), prepare one or more five-fold dilutions of the test sample. When testing **culture supernatants**, prepare the serial dilutions using the culture medium. When testing **serum or plasma,** prepare the serial dilutions using the provided Standard Diluent. For example, a five-fold dilution is prepared by adding 0.1mL (100µL) of test sample to 0.4mL (400µL) of appropriate diluent. Mix samples thoroughly between dilutions before assaying.

Reagent Preparation

For procedural differences when using partial plates, look for **(PP)** throughout these instructions.

Wash Buffer

1. Label a new glass or plastic 2L container "Wash Buffer." The 30X Wash Buffer may have a cloudy appearance.
2. Add entire contents of the 30X Wash Buffer (50mL) bottle to the 2L container and dilute to a final volume of 1.5L with ultrapure water. Mix thoroughly.

   (PP) When using partial plates, store the reconstituted Wash Buffer at 2-8°C.

Note: Wash Buffer must be at room temperature before use in the assay. Do not use Wash Buffer if it becomes visibly contaminated during storage.

Standards

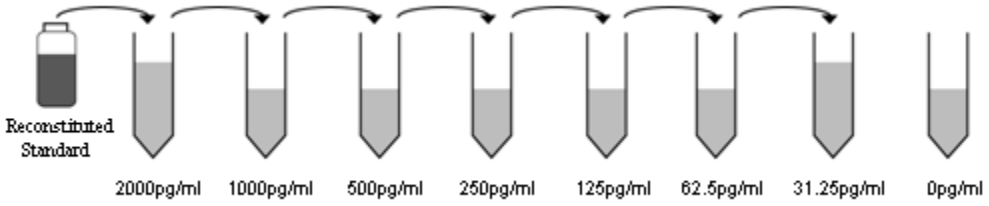
(PP) Reconstitute and use one vial of the lyophilized Standard per partial plate.

1. Prepare Standards just before use and use within one hour of reconstitution. Do not store reconstituted standards.
2. When testing **culture supernatant samples**, reconstitute Standard with Standard Diluent. Reconstitution volume is stated on the Standard vial label. The Standard will dissolve in ~1 minute. Mix by gently inverting vial. Use the sample culture medium to prepare standard curve dilutions.

   When testing **serum or plasma samples,** reconstitute Standard with Standard Diluent. Reconstitution volume is stated on the Standard vial label. The Standard will dissolve in ~1 minute. Mix by gently inverting vial. Use the Standard Diluent provided to prepare the standard curve serial dilutions.

   When testing **serum, plasma and cell culture supernatant samples on the same plate,** validate the media to establish if the same standard curve can be used for the different sample types. Prepare a standard curve (including a zero/blank) using culture medium to reconstitute and dilute the Standard. Use medium containing serum or other protein to maximize stability of the human VEGF. Perform this curve in parallel with a standard curve reconstituted in Standard Diluent and diluted in the Standard Diluent provided. If the OD values of the two curves are within 10% of the mean for both curves, then the assay can be performed with Standard Diluent, whether you are testing culture supernatant, plasma or serum samples.
3. Label eight tubes, one for each standard curve point: 2000, 1000, 500, 250, 125, 62.5, 31.25, and 0pg/mL. Prepare 1:2 serial dilutions for the standards as follows:
4. Pipette 250µL of appropriate diluent into all of the tubes.
5. Pipette 250µL of the reconstituted standard into the first tube (i.e., 2000pg/mL) and mix.
6. Pipette 250µL of this dilution into the second tube (i.e., 1000pg/mL) and mix.
7. Repeat the serial dilutions (using 250µL) five more times to complete the standard curve points. These concentrations, 2000, 1000, 500, 250, 125, 62.5, 31.25, and 0pg/mL are the standard curve.

Serial Dilutions using 250µL



Assay Procedure

A. Calibrator and Sample Incubation

- **(PP)** Determine the number of strips required. Leave these strips in the plate frame. Tightly seal remaining unused strips in the provided foil pouch with desiccant and store at 2-8°C. After completing the assay, retain the plate frame for the second partial plate. When using the second partial plate, place strips securely in the plate frame.
- Use the Plate Template provided at the end of this instruction book to record the locations of the zero standard (blank or negative control), human VEGF standards and samples. Perform seven standard points and one blank in duplicate with each series of unknown samples.
- If using a multichannel pipettor, use a new reagent reservoir to add the Biotinylated Antibody Reagent. Remove from the vial only the amount required for the number of strips being used.

1. Add 50µL of Standard Diluent to all wells.
2. Add 50µL of diluted standards and test samples to each well. Mix well by gently tapping the plate several times.
3. Carefully cover the plate with an adhesive plate cover. Ensure all edges and strips are tightly sealed by running your thumb over the edges and down each strip. Incubate for 2 hours at room temperature (20-25°C).
4. Carefully remove the adhesive plate cover. Wash plate THREE times with Wash Buffer as described in the Plate Washing Section (Section B).

B. Plate Washing

1. Gently squeeze the long side of the plate frame before washing to ensure all strips securely remain in the frame.
2. Empty plate contents. Use a squirt bottle to vigorously fill each well completely with Wash Buffer, then empty plate contents. Repeat procedure two additional times for a total of THREE washes. Blot plate onto paper towels or other absorbent material.

   Note: For automated washing, aspirate all wells and wash THREE times with Wash Buffer, overfilling wells with Wash Buffer. Blot plate onto paper towels or other absorbent material.

C. Biotinylated Antibody Reagent Incubation

1. Add 100µL of Biotinylated Antibody Reagent to each well.
2. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate the plate for 1 hour at room temperature (20-25°C).
3. Carefully remove the adhesive plate cover, discard the plate contents and wash THREE times as described in the Plate Washing Section.

D. Streptavidin-HRP Solution Preparation and Incubation

- Prepare Streptavidin-HRP Solution **just before use.** Do not prepare more solution than required.
- Do not store the prepared Streptavidin-HRP Solution.
- Use a 15mL plastic tube to prepare the Streptavidin-HRP Solution.
- Use a new reagent reservoir and pipette tips when adding the prepared Streptavidin-HRP Solution.

1. Briefly centrifuge the Streptavidin-HRP Concentrate to force entire vial contents to the bottom.
2. **(PP)** Use only the Streptavidin-HRP Solution amount required for the number of strips being used. For each strip, mix 2.5µL of Streptavidin-HRP Concentrate with 1mL of HRP Dilution Buffer. Store Streptavidin-HRP Concentrate reserved for additional strips at 2-8°C.
3. For one complete 96-well plate, add 30µL of Streptavidin-HRP Concentrate to 12mL of HRP Dilution Buffer and mix gently.
4. Add 100µL of prepared Streptavidin-HRP Solution to each well.

5. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate the plate for 30 minutes at room temperature (20-25°C).
6. Carefully remove the adhesive plate cover, discard the plate contents and wash THREE times as described in the Plate Washing Section.

E. Substrate Incubation and Stop Solution Addition

- Use new disposable reagent reservoirs when adding the TMB Substrate Solution and Stop Solution.
- From the bottle, dispense ONLY the required amount of 100µL per well for the number of wells being used. Do not use a glass pipette to measure the TMB Substrate Solution.
- **(PP)** Do not combine leftover substrate with that reserved for the second partial plate. Take care not to contaminate the remaining TMB Substrate Solution.

1. Pipette 100µL of TMB Substrate Solution into each well.
2. Allow color reaction to develop at room temperature in the dark for 30 minutes. Do not cover plate with aluminum foil or a plate sealer. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
3. After 30 minutes, stop the reaction by adding 100µL of Stop Solution to each well.

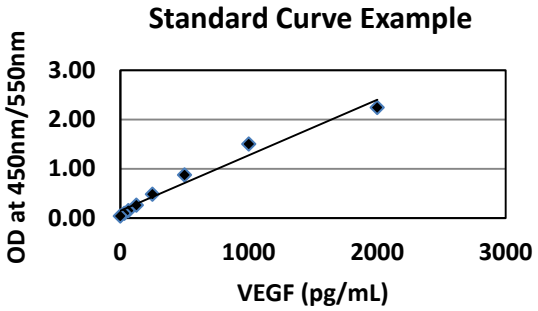
F. Absorbance Measurement

Evaluate the plate within 30 minutes of stopping the reaction. Measure absorbance on an ELISA plate reader set at 450nm and 550nm. Subtract 550nm values from 450nm values to correct for optical imperfections in the microplate. If an absorbance at 550nm is not available, measure the absorbance at 450nm only.

Note: When the 550nm measurement is omitted, absorbance values will be higher.

G. Calculation of Results

- The standard curve is used to determine human VEGFA amount in an unknown sample. Generate the standard curve by plotting the average absorbance obtained for each Standard concentration on the vertical (Y) axis vs. the corresponding human VEGFA concentration (pg/mL) on the horizontal (X) axis.
- Calculate results using graph paper or curve-fitting statistical software. Determine the human VEGFA amount in each sample by interpolating from the absorbance value (Y-axis) to human VEGFA concentration (X-axis) using the standard curve.
- If the test sample was diluted, multiply the interpolated value obtained from the standard curve by the dilution factor to calculate pg/mL of human VEGFA in the sample.
- Absorbance values obtained for duplicates should be within 10% of the mean value. Carefully consider duplicate values that differ from the mean by greater than 10%.



Performance Characteristics

Sensitivity: ≤ 5pg/mL
The sensitivity, or Lower Limit of Detection (LLD), was determined by assaying replicates of zero and the standard curve. The mean signal of zero + two standard deviations read in dose from the standard curve is the LLD. This value is the smallest dose that is not zero with 95% confidence.

Assay Range: 31.25-2000pg/mL
Suggested standard curve points are 2000, 1000, 500, 250, 125, 62.5, 31.25 and 0pg/mL

Reproducibility:
Intra-Assay CV: < 10%
Inter-Assay CV: < 10%

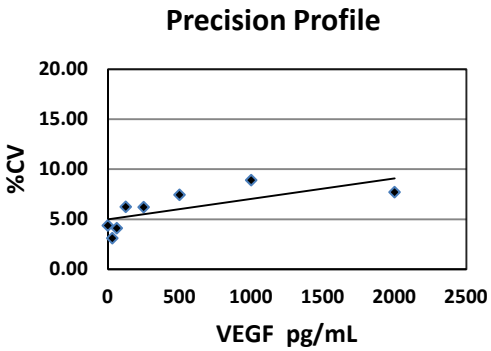
Specificity: This ELISA is specific for the measurement of natural and recombinant human VEGF. No significant cross-reaction with human IL-1A, IL-1B, IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-13, IL-16, IL-17, human GM-CSF, RANTES, PLGF-1, PLGF-2, VEGFB, VEGFC, VEGFD, mouse VEGF and rat VEGF.

Calibration: The standards in this assay were calibrated to the NIBSC reference standard lot 02/286; 2000pg/mL of internal standard equals 1290pg/mL of NIBSC reference standard.

Expected Values:

Cell Culture Supernatants: 293T cells were cultured in DMEM cell culture media with 10% fetal calf serum. Cultured 293T cells were transfected with 2µg of ready-to-use human VEGF expression clone with CMV promoter. Supernatant was collected after 48 hours of transfection and > 3ng/mL of human VEGFA was measured.

Precision: The standard curve's average coefficients of variation (CV) are plotted against VEGFA concentration (pg/mL). The points represent samples evaluated in replicates of two in 10 representative pre-coated plates.



Recovery: Five samples of various matrices type: Human serum, EDTA plasma and heparin plasma were initially diluted 1:3 with Sample Diluent, spiked with known concentrations of VEGFA and assayed to calculate mean % recovery.

	Mean % recovery		
Spiked concentration (pg/mL)	**Serum (n=5)**	**Plasma EDTA (n=5)**	**Plasma heparin (n=5)**
1000	106	84	104
500	104	93	97
250	99	95	91

Dilution Linearity: Five individual donor samples for each sample type: Human serum, EDTA plasma and heparin plasma were initially diluted 1:3 with Sample Diluent and spiked with a high concentration of VEGF. Samples were further serially diluted with appropriate Sample Diluent and assayed.

	Mean % recovery		
Dilution factor	**Serum (n=5)**	**Plasma EDTA (n=5)**	**Plasma heparin (n=5)**
1:2	112	99	101
1:4	104	96	97
1:8	103	94	93

General References

Immunoassay: A Practical Guide. Chan and Perlstein, Eds. (1987). Academic Press: New York. p.71.

Neufeld, G., *et al.* (1999). Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* **13**:9-22.

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Current product instructions are available at *www.thermoscientific.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.

Plate Templates

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